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Arbeit unter wissenschaftlicher Betreuung von
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Corneal and scleral permeability of Desmoteplase in different species

Inaugural Dissertation

zur Erlangung der Doktorwürde der
Vetsuisse-Fakultät Universität Zürich

vorgelegt von

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aus Basel, BS

genehmigt auf Antrag von

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2020

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Vetsuisse-Fakultät Universität Zürich (2020)

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Intraocular fibrin clots caused by uveitis can be a sight-threatening condition which needs to be resolved quickly. Intracameral injection of tissue-plasminogen activator (tPA) is commonly used to resolve intraocular fibrin. However, the drug does not reach fibrinolytic concentrations after topical application. Desmoteplase (DSPA) is a structurally similar but smaller fibrinolytic agent with a higher fibrin selectivity, a longer half-life, and better biocompatibility compared to tPA. This study was designed to evaluate the corneal and scleral permeability of DSPA in rabbits, pigs, dogs, horses and humans ex vivo.

Corneal and scleral tissues were exposed to DSPA in Franz-diffusion chambers. Drug concentrations on the receiver side were determined by liquid chromatography tandem mass spectrometry.

Concentrations of DSPA after corneal and scleral permeation through fresh tissues ranged from 0.0 to 16.3 µg/ml and 0.0 to 11.4 µg/ml (rabbits), 0.3 to 5.6 µg/ml and 3.1 to 9.2 µg/ml (dogs), 2.1 to 14.9 µg/ml and 4 to 8.7 µg/ml (horses), 0.6 to 3 µg/ml and 2.9 to 18.1 µg/ml (pigs), respectively. A concentration of 0.07 to 12.9 µg/ml DSPA was detectable after diffusion through tissue culture preserved human donor bank corneas.

DSPA has the ability to permeate both cornea and sclera ex vivo in all species tested. Implications of the ex vivo permeability of DSPA suggest that in vivo permeability may be possible, and if so it could lead to a novel topical application for lysing fibrin.

Keywords: permeability, desmoteplase, cornea, sclera

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Intraokuläres Fibrin, während einer Uveitis, kann das Auge nachhaltig schädigen. Deshalb ist eine schnelle Auflösung dieser Gerinnsel notwendig. Die intrakamerale Injektion von Gewebsplasminogenaktivator (t-PA) wird zur Auflösung solcher Gerinnsel eingesetzt, erreicht jedoch bei topischer Applikation keine fibrinolytisch wirksame Konzentration. Desmoteplase (DSPA) ist ein strukturell ähnliches, aber kleineres Molekül, welches eine höhere Fibrinselektivität, eine längere Halbwertszeit und eine bessere Bioverträglichkeit als t-PA aufweist.

Die Diffusion durch Kornea und Sklera wurden in Franz-Diffusionszellen getestet. Die erreichte Konzentration im Empfängermedium wurde mittels Flüssigkeitschromatographie mit Tandem-Massenspektrometrie bestimmt.

Durchschnittliche DSPA Konzentrationen (+/- Standardabweichung) nach Permeation durch Kornea und Sklera betrugen 0.0 bis 16.3 µg/ml und 0.0 bis 11.4 µg/ml (Kaninchen), 0.3 bis 5.6 µg/ml und 3.1 bis 9.2 µg/ml (Hunde), 2.1 bis 14.9 µg/ml und 4 bis 8.7 µg/ml (Pferde), 0.6 bis 3 µg/ml und 2.9 bis 18.1 µg/ml (Schweine). Eine Konzentration von 0.07 bis 12.9 µg/ml DSPA wurde nach Diffusion durch menschliche Hornhäute festgestellt.

DSPA diffundiert durch Gewebeproben aller getesteten Spezies *ex vivo*. Die Ergebnisse dieser *ex vivo* Studie implizieren die Möglichkeit einer Diffusion *in vivo* und könnten dadurch zur Entwicklung eines topisch applizierbaren Fibrinolytikums führen.

Stichworte: Permeabilität, Desmoteplase, Hornhaut, Sklera

Corneal and scleral permeability of Desmoteplase in different species

Corneal and scleral permeability of Desmoteplase

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Abstract

Purpose: Intracameral injection of tissue-plasminogen activator (tPA) is commonly used to resolve intraocular fibrin. The drug does not reach fibrinolytic concentrations after topical application. Desmoteplase (DSPA) is a structurally similar but smaller molecule, with a higher fibrin selectivity, a longer half-life and better biocompatibility compared to tPA. This *ex vivo* study was designed to evaluate the corneal and scleral permeability of DSPA in rabbits, pigs, dogs, horses and humans.

Methods: Corneal and scleral tissues (n=5 per group) were inserted into Franz-diffusion chambers and exposed to 1.4mg/ml DSPA for 30 min. Drug concentrations on the receiver side were determined by liquid chromatography tandem mass spectrometry.

Results: Concentrations of DSPA after corneal and scleral permeation ranged from 0.0 to 65.4 µg/ml and 0.0 to 45.6 µg/ml (rabbits), 1.4 to 22.3 µg/ml and 12.2 to 36.8 µg/ml (dogs), 8.4 to 59.4 µg/ml and 15.7 to 34.9 µg/ml (horses), 2.4 to 11.8 µg/ml and 11.4 to 72.3 µg/ml (pigs), respectively. 0.3 to 51.6 µg/ml DSPA was detectable after diffusion through human corneas (table 1).

Conclusions: DSPA has the ability to permeate cornea and sclera *ex vivo* in all species examined. The fibrinolytic capacity of DSPA after topical application needs to be tested *in vivo*.

1. Introduction

Acute, severe uveitis is often observed with fibrinous exudates and a subsequent fibrin clot formation is a common complication in both animals and humans. Persistence of fibrinous clots and strands can lead to synechiae or obstruct the iridocorneal angle and thus cause secondary glaucoma, decrease transparency of ocular media and ultimately cause vision loss [1, 2]. Early and efficient lysis of intraocular fibrin is therefore of great importance. Treatment with steroids or injections of genetically modified (i.e. recombinant) tissue-plasminogen activator (tPA) as a fibrinolytic agent are used to dissolve fibrin clots.

tPA is a direct plasminogen activator (PA), activating plasminogen by cleaving the Arg561-Val562 bond, resulting in catalytically active plasmin. tPA has low affinity to plasminogen but high affinity to fibrin. High concentrations of tPA and plasminogen, which also has a high affinity to fibrin, on the surface of fibrin clots lead to lysis of fibrin into soluble split products within approximately 2 hours of intracameral injection of tPA. However, tPA causes also limited conversion of plasminogen in the absence of fibrin which therefore could lead to systemic haemorrhage [3, 4].
Due to molecular characteristics, such as size (molecular weight (MW) of 70 kDa) and polarity, tPA does not permeate the cornea or sclera in concentrations sufficient to induce intraocular fibrinolysis after topical application [4-8]. Therefore, tPA must be applied via intraocular injection, which bears a risk of infection, increase of inflammation, bleeding, or inadvertent trauma [9]. Furthermore, the short tPA half-life of 5 minutes cannot prevent re-clotting in case of a persistent inflammatory state of the eye which can necessitate multiple injections [7, 8]. For intracameral doses exceeding 25 µg tPA, a dose-dependent toxic effect on retinal and endothelial cells was demonstrated *in vitro* and *in vivo* [6, 10-13].

Desmoteplase (DSPA), originally found in saliva of the strictly blood-feeding vampire bat species *Desmodus rotundus,* is a plasminogen activator, closely related to tPA. [7, 14]. DSPAα1, the most fibrin-specific agent of all four known molecular variants, was ultimately chosen for clinical development. Structurally, the molecule is 89% identical with the five classic tPA domains, but is the only PA known to exist exclusively as a single chain molecule with full catalytic activity, resulting in a smaller molecule size (52 kDa MW) [3, 14]. In contrast to tPA, DSPA is an indirect PA which only requires fibrin as cofactor for plasminogen activation, consequently granting strictly localized fibrinolytic activity with a minimized risk of haemorrhages elsewhere [3, 15-17]. DSPA has a 180-fold higher fibrin selectivity compared to tPA, which is based on the lack of a plasmin sensitive processing site present in tPA[7].
Compared to tPA's 5-minute half-life, DSPA has a longer half-life of approximately 3 hours.
Contrary to tPA, DSPA has shown no neurotoxicity *in vitro* or *in vivo* [14, 18-20]. Light microscopic evaluation of endothelial and retinal cell morphology at 400× magnification has revealed no identifiable signs of toxicity after an intracameral injection of DSPA into rabbit eyes [21]. Thus, DSPA could have advantages over tPA when used for intraocular fibrinolysis, especially in case of sufficient fibrinolytic efficacy after topical application.

Overcoming ocular barriers is an important issue when administering eye drops as only 1-7% of topically instilled drugs penetrate into the anterior chamber [22]. Nevertheless, topical administration is a preferred route of administration because it can avoid the traumatic insult, potential iatrogenic intraocular bacterial contamination, and the repeated need for sedation associated with intracameral injections [23].

Ocular permeability depends on several parameters. Precorneal factors (solution drainage, blinking, tear film composition and turnover) and anatomical barriers including the various layers of the cornea, conjunctiva and sclera as well as their thickness and surface area play an important role in drug permeation [22-26]. The cornea is considered to be the main route of permeation after topical application of drugs but larger molecules permeate the eye mainly by diffusion through the sclera [22, 24, 25, 27-30]. The epithelium with its intercellular tight junctions represents the first barrier to pass; hydrophilic, small molecules have been shown to permeate between cells, whereas the transcellular route contributes to epithelial transfer of lipophilic drugs. The epithelium is a rate-limiting barrier for highly polar, high-MW drugs and contributes 90% of the barrier for hydrophilic substances [22, 23]. The corneal stroma acts as a barrier to lipophilic molecules [26]. Endothelial junctions on the other hand are leaky and allow the passage of macromolecules [26]. Scleral permeability was found to be approximately 10 times higher than corneal permeability and is directly proportional to scleral thickness and total surface area [22]. Interestingly, globular proteins permeate the sclera better than linear dextrans, which makes the molecular radius of the applied compound a better predictor of scleral permeability than the MW [22]. Also, lipophilic or positively charged molecules have shown worse scleral permeability than hydrophilic or negatively charged molecules [26]. Passive diffusion is the primary route forsolute transport and compounds up to 150 kDa MW are able to penetrate the sclera [22]. The equatorial region of the sclera is significantly thinner and consequently more permeable to macromolecules than the posterior segment [28, 30]. Considering the molecular size of DSPA, intraocular permeation was expected to occur primarily via scleral diffusion.

For this reason, both transcorneal and transscleral diffusion of DSPA was investigated in *ex vivo* rabbit, canine, equine, porcine, and human tissues in this study.

2. Materials and methods

Tissue preparation

Corneal and scleral tissues of rabbit, porcine, equine and canine eyes, and human corneal tissues were collected for experimentation. Fresh rabbit, porcine and equine eyes were obtained from registered slaughterhouses in Switzerland. Canine samples were collected with owner consent from animals euthanized due to reasons unrelated to the study. All eyes were examined with a focal light source to ensure that a normal corneal surface and anterior chamber were present. Corneal de-epithelialization

was avoided [31]. The globes were stored in Hanks Balanced Salt Solution (HBSS) [32] (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland), at 4°C and processed within 4 hours after death. Rabbit, porcine, and canine corneas were dissected at the limbus, 10 mm skin biopsy punches (Acu Punch, Acuderm, Fort Lauderdale, USA) were used to excise central corneal buttons from the equine eyes as well as scleral buttons from all animal species.

Human corneas were obtained from the donor bank of Inselspital Bern (Bern, Switzerland; donor corneas not fitting transplantation criteria). No human scleral tissue was available for this study. The human corneas had been stored in Minimum Essential Medium (MEM), 2% foetal bovine serum and 1% antibiotic solution [33] at 36°C and 5% CO_2, and were immersed prior to the experiment in MEM + 6% dextran solution at 36°C for at least 24 hours to reduce both tissue swelling and volume.

DSPA concentration

For the present study, a donor volume of 300 µl and a receptor volume of 5 ml were used. A drug permeation of 1% was considered based on the previously reported 1-7% permeation of topically instilled drugs into the anterior chamber [22]. An intravascular concentration of ≥0.86 µg/ml DSPA was determined to lyse intravascular clots most efficiently in a prior study [34]. Therefore, a concentration of 1.4 mg/ml DSPA was chosen for the experiment, translating to 420 µg DSPA in 300 µl donor fluid, as this would lead to a concentration of 0.84 µg/ml DSPA (4.2 µg/5ml) in the receiver compartment.

Permeation experiments

DSPA was obtained from Lundbeck (Valby, Denmark). DSPA permeation experiments in Franz-type diffusion cells were performed as described previously (Fig. 1; SES GmbH – Analysesysteme, Bechenheim, Germany) [24, 35].

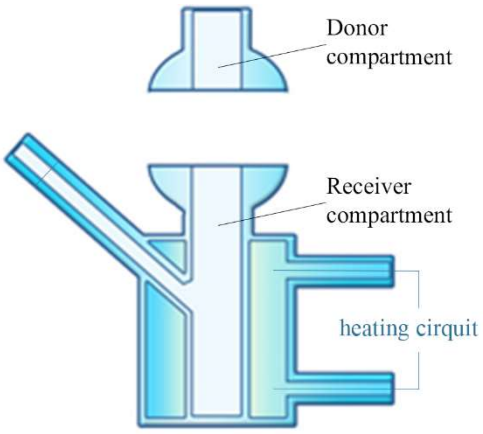

Fig. 1 Franz diffusion chamber schematic

The donor compartment was filled with 300 µl of 1.4 mg/ml DSPA (pH 7.5, 0.027 mmol/l) or Hepes buffer for negative controls only (n=2, rabbit cornea and sclera). The receiving phase consisted of 5 ml 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes buffer, 10 mM, pH 7.4, 140 mM NaCl) [12], thermostated at 37°C and stirred with a magnetic bar. Tissues were carefully mounted, avoiding remaining air bubbles to optimize the liquid-tissue contact zone (0.2 cm^2).

Analytical Method

After 30 minutes of sample exposure to the donor phase solutions, 1 ml samples were collected from the receiver compartment [27]. Aliquots were taken for analysis and the remaining sample volume was frozen at -20°C for future use. The concentrations of DSPA in the receiver compartment solution were initially determined via ultraviolet visible spectrometry at 280 nm wavelength, as specified for DSPA patent WO2011107299A2. Due to positive ultraviolet visible spectrometry measurement values of blank controls at 280 nm this method was considered as insufficiently specific for DSPA.

The frozen samples (-20°C) were sent to the Functional Genomics Center Zurich for absolute quantitation of DSPA by liquid chromatography - tandem mass spectrometry (LC-MS/MS) using parallel reaction monitoring (PRM): To identify peptides for PRM, DSPA samples were digested with trypsin and then analysed. Detected peptides were searched against the SwissProt database using Skyline [36] and the only suitable peptide (DSPA138-146 (GTWSTAESR) was synthesized with $6^{13}C4^{15}N$-Arg (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland).

All samples were precipitated with an equal volume of 20% trichloroacetic acid (TCA; Sigma-Aldrich) and washed twice with cold acetone. The dried pellets were digested with trypsin in a microwave instrument (Discover System, CEM) for 30 min at 5 W and 60°C. Digested samples were dried again and dissolved in 40 µl 0.1% formic acid (Romil, Cambridge, United Kingdom) containing the labeled DSPA138-146 (20 fmol/µl). Samples were analyzed by LC-MS/MS on a nanoAcquity UPLC (Waters Inc., Milford, USA) connected to a Q Exactive mass spectrometer (Thermo Fisher Scientific). Set masses for the PRM were m/z 497.7331 and 502.7331 for the light and heavy peptide, respectively.

The limit of detection (LOD) of the DSPA138-146 peptide was determined to be < 0.13 pmol corresponding to about 6.25 pg of DSPA injected onto the LC column. The limit of quantitation (LOQ) was in the range of 1 – 2 fmol DSPA138-146 peptide (about 50 to 100 pg DSPA) on column. The linearity interval ($R^2 = 0.99$) ranged from 1 to 80 fmol (about 50 pg – 4 ng DSPA) on column.

Data Processing

Absolute concentrations of DSPA were calculated in Skyline [37] by integration of the parent ion signal of the peptide DSPA138-146 using the known amount of the synthetic AQUA peptide as internal standard.

Statistical Analysis

A sample size of n=5 per group was recommended by biostatisticians of the Vetsuisse Faculty (detection of disease principle) to provide a ‘yes or no’ answer to the question whether any diffusion of DSPA could be measured across corneal and/or scleral tissues. The *ex vivo* study was designed to

determine whether *in vivo* experiments evaluating the efficacy of topically applied DSPA would be justified. Concentrations of DSPA within an eye were assessed using descriptive statistics.

3. Results

Concentrations of DSPA after corneal and scleral permeation ranged from 0.0 to 16.3 µg/ml and 0.004 to 11.4 µg/ml (rabbits), 0.3 to 5.6 µg/ml and 3.1 to 9.2 µg/ml (dogs), 2.1 to 14.9 µg/ml and 4 to 8.7 µg/ml (horses), 0.6 to 3 µg/ml and 2.9 to 18.1 µg/ml (pigs), respectively. A concentration of 0.07 to 12.9 µg/ml DSPA was detectable after diffusion through human corneas (Table 1).

Table 1: Concentrations of DSPA in the samples from the receiver compartment

tissue	**Concentration in fmol/ µl**	**concentration in µg/ml**	**tissue**	**concentration in fmol/µl**	**concentration in µg/ml**
rabbit cornea	0.305	16.328	rabbit sclera	0.213	11.396
	0	*0*		0.059	3.136
	0.162	8.669		0.149	7.981
	0.167	8.933		0	*0.004*
	0.035	1.875		0.04	2.169
dog cornea	0.08	4.307	dog sclera	0.117	6.262
	0.016	*0.866*		0.075	4.025
	0.006	*0.339*		0.099	5.331
	0.081	4.339		0.161	8.644
	0.034	1.801		0.057	3.045
	0.104	5.571		0.172	9.191
pig cornea	0.011	*0.603*	pig sclera	0.072	3.833
	0.051	2.719		0.337	18.085
	0.015	*0.784*		0.053	2.862
	0.055	2.952		0.122	6.553
	0.02	1.087		0.203	10.889
	0.019	0.996		0.079	4.216
horse cornea	0.111	5.932	horse sclera	0.073	3.937
	0.103	5.495		0.161	8.604
	0.277	14.861		0.117	6.295
	0.093	4.997		0.095	5.098
	0.039	2.101		0.161	8.639
	0.163	8.722		0.163	8.725
human cornea	0.012	*0.638*			
	0.241	12.903			
	0.001	*0.074*			
	0.004	*0.204*			
	0.003	*0.171*			

4. Discussion

Our study documents a successful measurement of DSPA after diffusion through ocular tissues of different species in Franz-diffusion chambers. The study was conducted as a proof of principle study for ethical reasons; in case of negative results, a following *in vivo* study would not have included topical application of DSPA [21]. To the authors' knowledge, this is the first report evaluating the permeation of DSPA through corneal and scleral tissues. Permeation of DSPA through the cornea and sclera of rabbits, pigs, dogs and horses was demonstrated *ex vivo*, permeation through long-time stored human corneas was most likely influenced by tissue decay. The small number of samples did not allow statistical analysis of differences in DSPA permeation between tissues and species.

The first attempt to measure DSPA via UV/VIS-spectrometry was discarded since positive DSPA measurements were also obtained from negative control samples that had not been exposed to DSPA. We concluded that corneal and scleral tissue molecules of similar size to DSPA must have influenced the results, proving the UV/VIS-spectrometry method inappropriate for measuring DSPA diffusion across tissues. Liquid chromatography - tandem mass spectrometry is a very sensitive as well as compound-specific measurement method that was able to determine very small amounts of DSPA. Concentrations of DSPA could be determined using a DSPA specific heavy amino acid sequence to identify DSPA molecules. Blank samples demonstrated negative results when using this technique.

Permeation of DSPA was observed through both cornea and the sclera. Higher DSPA concentrations were expected after diffusion through scleral compared to corneal tissue on the grounds of DSPAs molecular size and the fact that larger molecules permeate the eye mainly by scleral diffusion[22]. The current study was not able to support this assumption, which may be explained by the small number of samples. Corneal and scleral thickness (rabbit cornea 348-387 µm [38], rabbit sclera 389.4 ± 4.95 µm [39], canine cornea 606 ± 7.4 µm [40], canine sclera 0.34 ± 0.13 mm [28], equine cornea 812.0 ± 44.1[41], equine sclera 0.53 ± 0.1 mm [28], human cornea 560-574 µm [42], human sclera 491 ± 91 µm [43]) as well as surface area vary between species. The results of drug permeability studies can therefore not be extrapolated from one species to others [24, 25, 28, 44, 45]. Nevertheless, we expected lower drug permeation in species with thicker tissues and higher values in species with a thinner cornea and sclera. However, those expected trends were not confirmed in this study. The variability across the *ex vivo* drug diffusion measurements might be explained by the small study sample size, combined with individual variability in ocular surface barrier anatomy and physiology [45].
Interestingly, the permeation values obtained through the thicker equine or porcine tissues were comparable or even higher than in the other species tested. Precise mounting of 10 mm diameter samples was challenging with the thick equine tissues. Correct tissue placement was checked after each diffusion experiment. All tissue samples remained in place during the experiment, completely covering the opening of the receiver compartment. Only with the relatively thick equine cornea a mild

fold in one place of the corneal button was detected in 2/5 specimens. Contamination of the receiver compartment with DSPA bypassing the tissue button despite careful sample placement can therefore not be excluded completely. A larger button size (>10 mm) and permeation testing of an inert dye as a control would therefore be recommended for future studies.

The extrapolation of results from *ex vivo* studies to an *in vivo* situation has been reported to be difficult because conjunctival and choroidal blood circulation influence tissue drug concentrations and therefore permeation of drugs in *in vivo* situations [45].
Equally, the application of DSPA over a 30-minute timeframe did not mirror an *in vivo* treatment situation as tear and aqueous humor turnover, conjunctival absorption, and other factors significantly influence drug concentrations *in vivo*. The concentration gradient of proteins in the eye thus differs from concentration gradients in the diffusion chamber. A recent review has described variable reproducibility of *ex vivo*-gained corneal penetration results in *in vivo* situations using different model systems. According to the authors of this review, diffusion cells were likely to overestimate transcorneal drug diffusion since the entire cornea is bathed in the drug solution, which can result in tissue overhydration and decreased corneal barrier properties [45]. *In vivo,* topically administered drugs have a short retention time of 5 minutes that could significantly decrease drug permeation compared to the *ex vivo* situation-[46, 47]. To reach similarly high drug concentrations in the same time, a multiple drug application regimen should be applied in an *in vivo* follow-up study.
Despite the difficulties in mimicking an *in vivo* situation, the 3R principle (replacement, reduction, refinement [48]) of laboratory animal experiments and animal welfare require exploitation of *ex vivo* systems first to justify intended *in vivo* trials.

This study suggests that clinically relevant intraocular concentrations might be reached via topical DSPA application in all species tested. Future studies should be designed to test whether permeation enhancers or subconjunctival injections could increase diffusion of DSPA. The intraocular bioavailability and efficacy of a topically administered drug depend on several factors, including tear film and aqueous humour turnover rates, which cannot be evaluated in an *ex vivo* drug permeation study [5, 26]. Clinical efficacy remains uncertain since the correlation between *ex vivo* and *in vivo* drug permeation study results is variable [45].

5. Acknowledgements

- Monika Kilchenmann for providing the human corneas.
- Lars Østergaard Pedersen & Peter Flagstad for providing the DSPA.
- Yolanda Joho-Auchli and Simone Wüthrich for the DSPA concentration measurements.
- Fernando Lagunas for bringing up the idea of testing DSPA.
- David Goldblum for his valuable recommendation on the experimental design.
- Sonja Hartnack for giving her input regarding statistical analysis and sample size.

6. Disclosures

The Authors declare that there is no conflict of interest.

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8. Figure Legend

Fig. 1: Franz diffusion chamber schematic

9. Table Legend

Table 1: Concentrations of DSPA in the samples from the receiver compartment

Danksagung

Ich danke den Mitarbeitern der Ophthalmologischen Abteilung des Tierspitals Zürich für Ihre fachliche und psychologische Unterstützung, sowie auch den Mitarbeitern des Physiologielabors der Vetsuisse Fakultät Zürich. Speziell erwähnen möchte ich Julia Baumann an dieser Stelle, für die wunderbare Einarbeitung und Hilfe.

Natürlich geht der Dank auch an meine beiden Betreuer:
Dr. med. vet. Katrin Voelter und Prof. Simon Pot

Ich danke aber vor allem meinen Eltern für die unendlichen Möglichkeiten und Unterstützung, die sie mir seit jeher gegeben haben.

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